Available online at www.sciencedirect.com

ScienceDirect

Virology 370 (2008) 392–402

VIROLOGY

www.elsevier.com/locate/yviro

The transcription strategy of bovine adeno-associated virus (B-AAV) combines features of both adeno-associated virus type 2 (AAV2) and type 5 (AAV5)

Chaoyang Ye, David J. Pintel *

Department of Molecular Microbiology and Immunology, University of Missouri-Columbia, School of Medicine, 471f Life Sciences Center, 1201 E. Rollins Rd., Columbia, MO 65211-7310, USA

Received 19 July 2007; returned to author for revision 30 August 2007; accepted 7 September 2007
Available online 17 October 2007

Abstract

The parvoviruses bovine adeno-associated virus (B-AAV) and adeno-associated virus type 5 (AAV5) have similar transcription maps. However, while the AAV5 capsid gene promoter P41 possesses a high basal level in 293 cells, and is further activated only poorly by Rep during adenovirus type 5 (Ad5) infection, the B-AAV P41 promoter has a low basal activity within RepCap constructs in these cells and can be strongly activated by its Rep protein in the presence of Ad5 when a Rep-binding element (RBE) is included in *cis* at either end of the molecule. These differences are not due to differences in the intrinsic activating capability of the individual Rep proteins. Both viral promoters contain AP1 and CRE elements that contribute to their basal activity; however, the nature of the B-AAV P41 promoter itself and the surrounding sequences contribute to its relatively lower basal activity. In addition, the B-AAV upstream transcription units themselves also are activated in the presence of Ad5 and Rep. Thus, although the transcription map of B-AAV is much more closely related to AAV5, activation of its promoters is functionally more like the prototype AAV2.

Keywords: Adeno-associated viruses; Transcription profile; AAV5; B-AAV; Promoter regulation

Introduction

As members of *Dependovirus* genus of the *Parvoviridae* family, adeno-associated viruses (AAVs) depend on helper viruses such as adenovirus type 5 (Ad5) or herpes simplex virus (HSV) to complete their life cycle. Different AAVs utilize diverse strategies to modulate their capsid promoter activity (Bowles et al., 2006; Cotmore and Tattersall, 2006). The AAV2 capsid promoter P40 has been extensively studied and, similar to many human AAVs, has a low basal transcription activity. In the presence of helper viral functions, P40 is activated strongly by its own large Rep protein following binding to a platform provided by its inverted terminal repeats (ITR) or P5 promoter (McCarty et al., 1991). The AAV5 capsid promoter P41, on the other hand, has a higher basal activity in Ad5 E1A- and E1B-expressing 293 cells, partly due to the presence of AP1 and CRE transcription factor binding sites upstream of the promoter, and is further activated only poorly by its Rep protein in the presence of Ad5 (Ye and Pintel, 2007).

AAV5 is the most divergent serotype of all human AAVs (Bantel-Schaal et al., 1999; Chiorini et al., 1999a,b) and its transcription organization is closely related to animal AAVs, such as caprine AAV (Go.1-AAV) and bovine AAV (B-AAV), which were originally isolated from animal adenovirus stocks (Arbetman et al., 2005; Clarke et al., 1979; Luchsinger et al., 1970; Myrup et al., 1976). For these viruses, the RNAs encoding the Rep proteins are primarily polyadenylated at a site in the center of the genome—a feature not utilized by the other human AAVs including AAV2 (Qiu et al., 2006a,b, 2005). The nucleotide sequence of AAV5 and B-AAV is more than 77% identical, which is greater than the homology between AAV5 and the other main serotypes of human AAV (AAV1, 2, 3, 4 and 6, which are all below 60%). Examination of the B-AAV P41 capsid promoter region revealed the presence of both AP1 and CRE sites (Ye and Pintel, 2007), yet in contrast to AAV5, its basal activity was low, and it was still significantly activated by

* Corresponding author. Fax: +1 573 884 9676.
E-mail address: pinteld@missouri.edu (D.J. Pintel).

0042-6822/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2007.09.007

B-AAV Rep targeted to the transcription template in the presence of Ad5 (Qiu et al., 2006b). This observation was unexpected given the high homology between AAV5 and B-AAV promoter region sequences, and their similar genetic organization. Considering these differences, further detailed comparison between AAV5 and B-AAV is called for. Additionally, because the RepCap constructs analyzed in the previous reports were built in slightly different backgrounds [B-AAV but not AAV5 constructs contained [1/2] ITR elements at both ends of the genome (Qiu et al., 2006b)], in this study, we systematically compared the transcription profiles of the two viruses under identical conditions. The activation of the B-AAV P41 promoter by its own Rep and interestingly, by AAV5 Rep as well, was confirmed. Although mutation of both the B-AAV P41 AP1 and CRE sites somewhat decreased its basal activity, the overall lower basal level of B-AAV P41 could be attributed to a combination of an intrinsic weakness in the promoter itself, and context-dependent effects of surrounding sequences. In linear B-AAV constructs with RBE-containing [1/2] ITR sequences at their left-hand end, upstream promoters (ITR, P7 and P19) were also activated in the presence of Ad5 and Rep. Thus, although B-AAV is much more closely related to AAV5, activation of its promoters is governed differently, and is functionally more like the prototype AAV2. These results demonstrated that B-AAV utilizes a combination of transcription strategies employed by both AAV2 and AAV5, and revealed an additional means employed by AAVs for governing their capsid gene expression.

Results

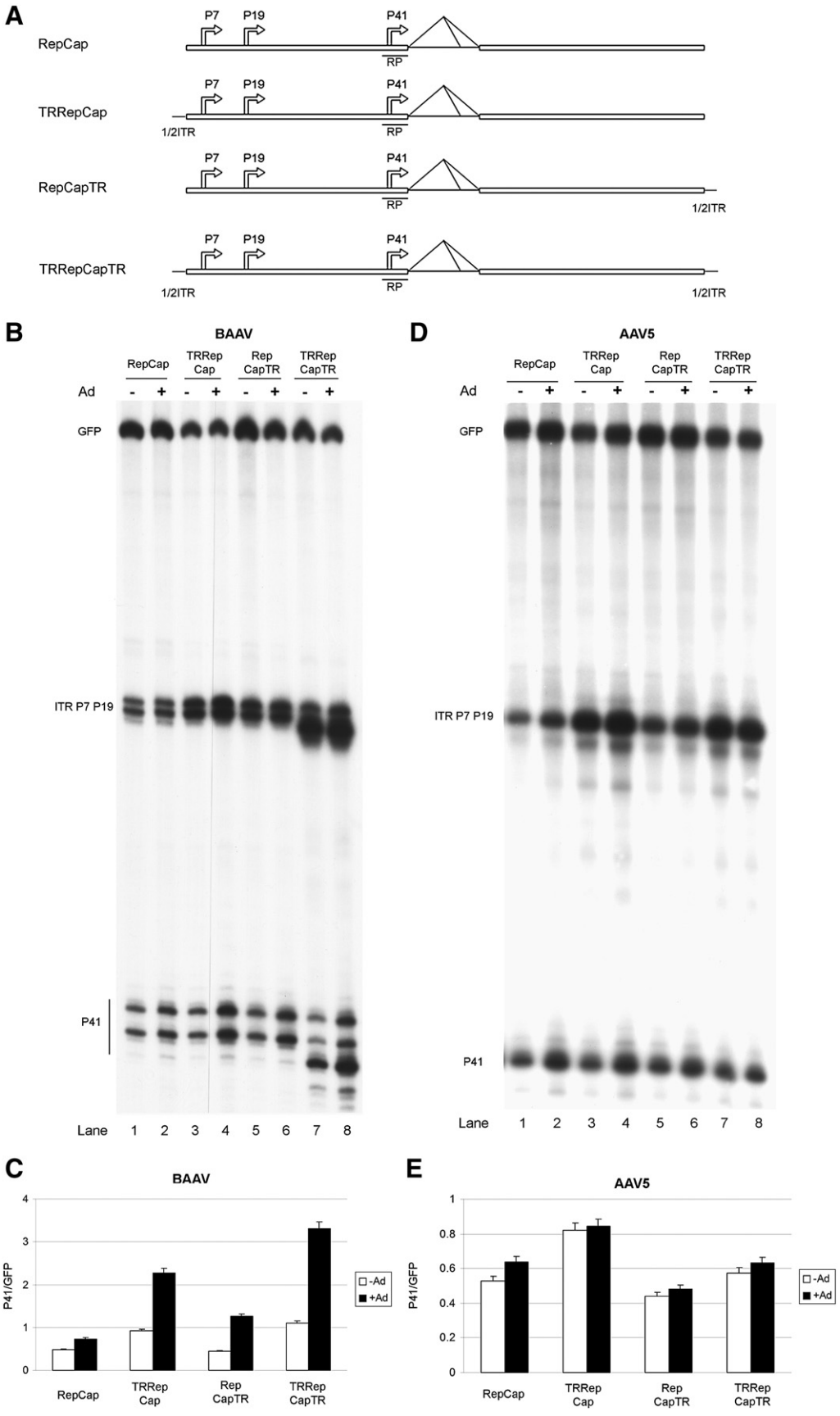
RNA expression profile differences between B-AAV and AAV5

Previously we have shown that, in E1A- and E1B-expressing 293 cells, the AAV5 capsid gene P41 promoter within ITR-deleted, noninfectious AAV5 RepCap plasmids exhibited a high basal level of transcription which was not further increased by helper Ad5 infection (Ye and Pintel, 2007). In addition, the AAV5 Rep protein was shown to be only a modest activator of P41 in 293 cells when a binding site was linked to the promoter (Ye et al., 2006). This is in contrast to the expression of the AAV2 P40 promoter, which in analogous constructs exhibits a very low basal activity which could be strongly activated by AAV2 Rep (Ye et al., 2006). Expression of the B-AAV P41 promoter in similar constructs, however, was low in 293 cells either in the absence or presence of Ad5, and when an RBE was introduced into this construct, in the form of a [1/2] ITR sequence, the activity of B-AAV P41 was significantly increased by its own Rep protein (Qiu et al., 2006b). This result was unexpected because the B-AAV nucleotide sequence and genetic organization are much more similar to AAV5 than to other human AAVs, yet its activation pattern was more similar to that of AAV2. Therefore, we decided to comprehensively compare the transcription of the B-AAV and AAV5 P41 promoters by placing them in a similar background, as diagrammed in Fig. 1A, and examining them in the same cell type. Examination of B-AAV in bovine cells is prevented currently because all available stocks of bovine adenovirus are contaminated with B-AAV. Our initial attempts to purify the stocks were unsuccessful and infection by human Ad5 in these cells was restricted (Qiu et al., 2006b). However, we have previously shown that B-AAV grows productively in 293 cells in the presence of human Ad5 (Qiu et al., 2006b), which afforded a permissive system for both viruses for our analysis. In addition to characterizing the ITR-deleted RepCap constructs previously described (Qiu et al., 2006b), we also extended the constructs at the 5′ and/or 3′ end so that an RBE-containing [1/2] ITR sequence was added. This provided a Rep-binding platform, yet still lacked essential *cis*-elements required for genome replication. For both sets of plasmids, P41 transcription levels in the presence or absence of Ad5 infection were compared using RNase protection assays and the quantification of these results is shown in the bar graph in Figs. 1C and E.

For B-AAV, P41 transcription from RepCap plasmids was only slightly increased by Ad5 infection (Fig. 1B, compare lane 2 to 1; Fig. 1C). Consistent with previous observations (Qiu et al., 2006b), more significant activation during Ad5 co-infection was observed from constructs with the RBE-containing [1/2] ITR sequence present, regardless of its position (2–3-fold, Fig. 1B, compare lanes 4, 6, and 8 to lanes 3, 5, and 7, respectively; Fig. 1C). As seen previously (Ye et al., 2006), however, P41 transcription from AAV5 was not significantly increased by Ad5 infection in any of the constructs (Fig. 1D, compare lanes 2, 4, 6, and 8 to lanes 1, 3, 5, and 7, respectively; Fig. 1E). Therefore, although genetically closer to AAV5, the transcription pattern of B-AAV was more similar to that of AAV2, whose large Rep proteins bind a RBE and recruits transcription factors for capsid promoter activation (Pereira and Muzyczka, 1997). Based on our previous comparison study between AAV2 and AAV5 (10), this could have been due to either differences in the activation ability of the two Rep proteins (Ye et al., 2006), and/or the nature of the *cis* sequences around the capsid promoters themselves (Ye and Pintel, 2007). Additionally, for both B-AAV and AAV5, the upstream transcripts from constructs containing left-hand [1/2] ITR sequences (TRRepCap and TRRepCapTR) were significantly more abundant than those from RepCap and RepCapTR.

B-AAV and AAV5 Rep have similar activation capabilities

AAV5 and B-AAV Rep proteins display nearly 90% amino acid identity. To functionally compare the potential roles of the B-AAV and AAV5 Rep proteins in P41 activation, we engineered a set of reporter plasmids (RepStopCap constructs), based on the RepCap constructs mentioned above, which no longer expressed Rep due to the introduction of premature termination codons downstream of the P7 promoters (diagrammed in Fig. 2A). The ability of AAV5 and B-AAV Rep proteins to activate transcription from the reporter plasmids was then assayed when provided *in trans* together with Ad5 infection. As seen previously, all B-AAV RepStopCap constructs, regardless of the placement of the RBE-containing [1/2] ITR sequence, produced very low levels of P41 transcripts in the absence of Rep proteins (Fig. 2B, lanes 1, 4, 7, and 10; Fig. 2C, open bars). Introduction of either AAV5 or B-AAV Rep did not increase expression of B-AAV P41 in the B-AAV RepStopCap plasmid due to the lack of Rep-binding sequences in this construct (Fig. 2B, compare lanes 2 and 3 to


A
RepCap
TRRepCap
RepCapTR
TRRepCapTR
P7
P19
P41
RP
1/2ITR
B
BAAV
RepCap
TRRep Cap
Rep CapTR
TRRep CapTR
Ad
GFP
ITR P7 P19
P41
Lane 1 2 3 4 5 6 7 8
D
AAV5
C
BAAV
P41/GFP
-Ad
+Ad
E
AAV5

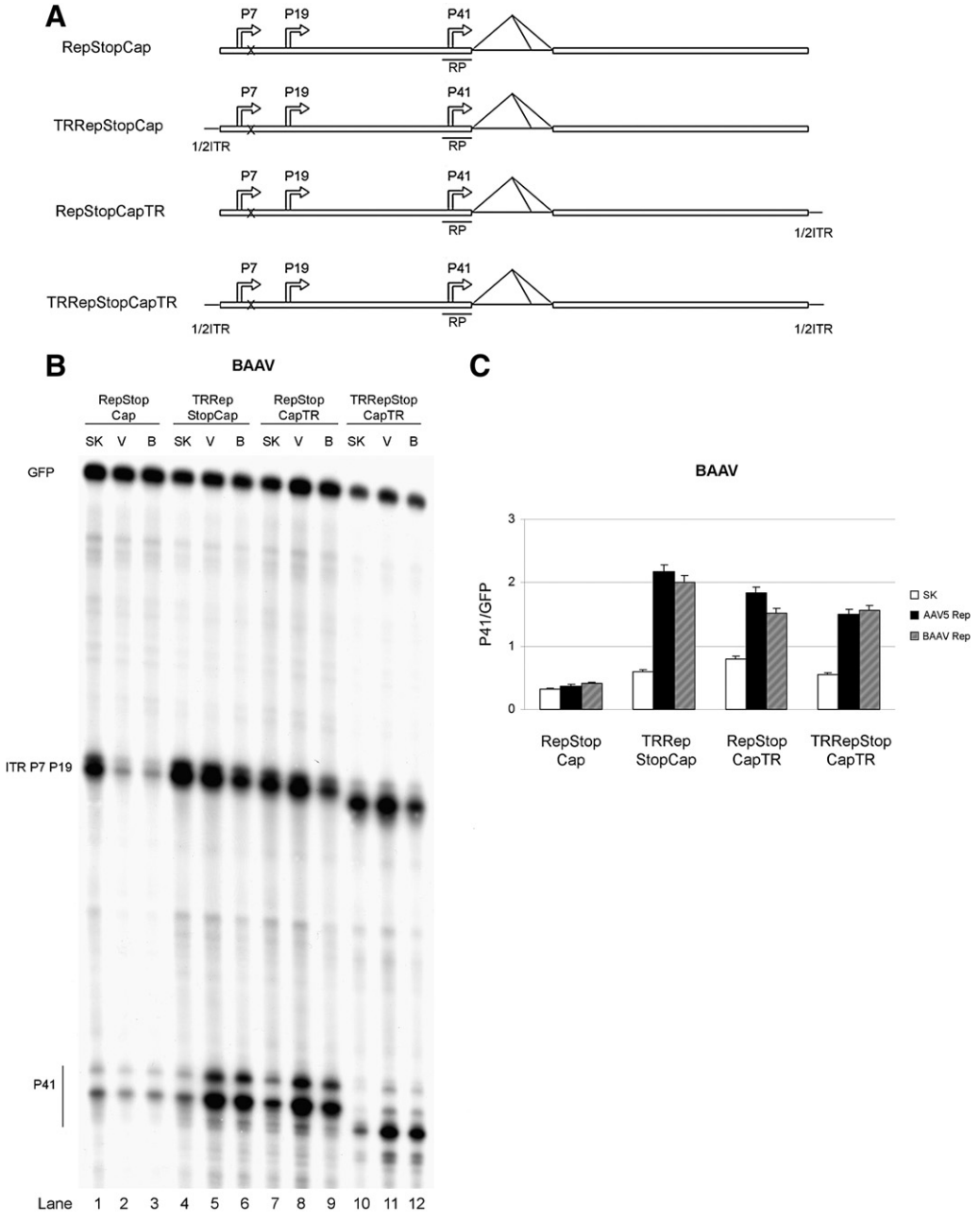
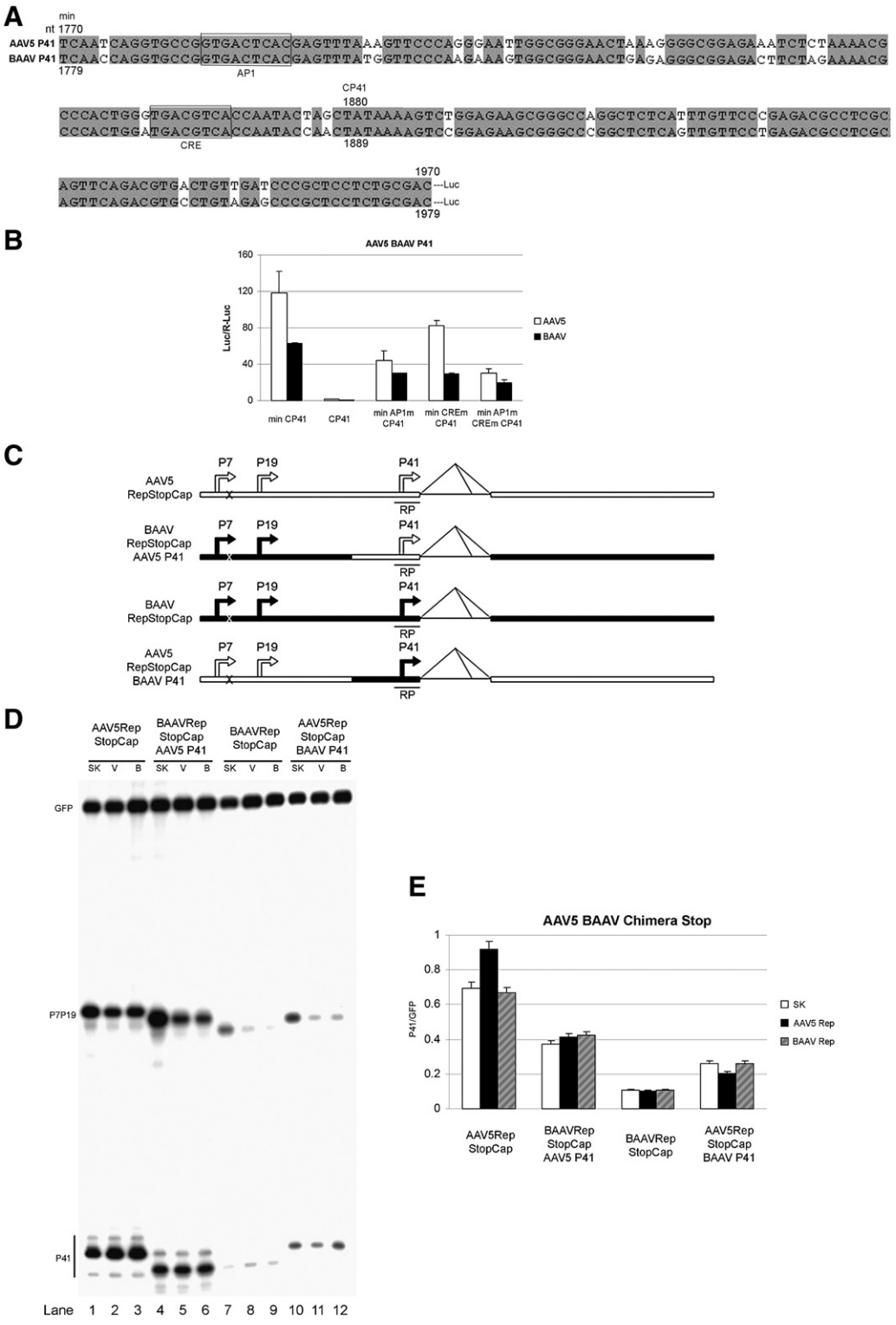


Fig. 2. Transcription activation by AAV5 and B-AAV Rep protein. (A) Diagrams of RepStopCap constructs and probes used in this experiment. Constructs are the same as in Fig. 1A except the introduction of premature termination codon at nt 541 for B-AAV (Qiu et al., 2006b). (B) RNase protection assay of the constructs co-transfected with pBluescript SK empty-vector control (SK) or AAV5 (V) or B-AAV Rep (B) expression constructs (Qiu et al., 2006b, 2002) as activators, followed by Ad5 infection. pEGFPC1 was co-transfected as control. GFP transcripts, and transcripts generated from upstream promoters (ITR+P7+P19), and P41 are labeled. (C) Quantification of the protection shown above. Data from at least three experiments, with standard error bars, are presented as the level of P41 transcripts normalized to GFP signal.

Fig. 1. RNA expression profile differences between B-AAV and AAV5 in response to Ad5 infection. (A) Diagrams of RepCap constructs used in this experiment. P7, P19, and P41 promoters are indicated by arrows. Probes across P41 region used in the RNase protection assay are labeled as RP (spanning nt 1855 to 1995 of B-AAV and 1846 to 1986 of AAV5). [1/2] ITR sequences were placed at the left (nt 81 to 192 of B-AAV and 75 to 184 of AAV5) or right hand (nt 4494 to 4637 of B-AAV and nt 4449 to 4591 of AAV5) of the genome to allow Rep binding. (B and D) RNase protection assay, performed as previously described (Naeger et al., 1992; Schoborg and Pintel, 1991), using the aforementioned RP probe across the P41 initiation site of RNA generated in 293 cells following transfection of B-AAV (B) or AAV5 (D) constructs diagramed in panel A. Lanes 7 and 8 have slightly faster migrating bands due to the mismatch at the end of the RP probe generated during the cloning procedure. pEGFPC1 (Clontech, Mountain View, CA) was co-transfected as internal control. Transcription signals from GFP, upstream promoters (ITRP7P19) and P41 are labeled. (C and E) Quantification, using Fujifilm MultiGauge software, of RNase protections, a representative of which is shown. Data from at least three experiments, with standard error bars, are presented as the level of P41 transcripts normalized to GFP signal.

lane 1; Fig. 2C). However, in constructs into which [1/2] ITR sequences competent for Rep binding were introduced (TRRepStopCap, RepStopCapTR, and TRRepStopCapTR), P41 transcription was significantly increased following co-transfection of either AAV5 or B-AAV Rep (Fig. 2B, compare lanes 5 and 6 to lane 4, lanes 8 and 9 to lane 7, and lanes 11 and 12 to lane 10, respectively; Fig. 2C). These results demonstrated that the basal activity of the B-AAV P41 promoter was low, and confirmed the role of these Rep proteins as activators of the B-AAV P41 promoter. Both Rep proteins were expressed at similar levels (data not shown). The relatively similar activation fold obtained suggested that AAV5 and B-AAV Rep have similar activation capabilities, and that activation by AAV5 Rep was apparent in this situation because of the low basal activity of B-AAV P41.



Cis sequences that govern the low transcription level of B-AAV capsid promoter

Sequence alignment revealed high identity between the B-AAV and AAV5 P41 promoter upstream sequences (Fig. 3A), with AP1 and CRE sites present in both. We have previously shown that disruption of the AP1 and CRE sites in the AAV5 P41 upstream region debilitated basal promoter activity, yet allowed a higher activation fold by AAV5 Rep (Ye and Pintel, 2007). Assay of luciferase reporter genes driven by different versions of the B-AAV and AAV5 P41 promoters showed that the upstream sequence of B-AAV functioned similarly to that of AAV5, increasing promoter activity significantly over the core promoter sequences alone (Fig. 3B, compare minCP41 to CP41)—although the overall promoter activity of B-AAV P41 remained lower than AAV5 P41. Mutation in the AP1 or CRE sites decreased the promoter activity to about half of the wild-type level (Fig. 3B, compare minAP1mCP41, minCREmCP41 to minCP41), and the double mutant promoter had an even lower activity (Fig. 3B, compare minAP1mCREmCP41 to minAP1mCP41 and minCREmCP41), consistent with what we observed previously for the AAV5 P41 promoter. Therefore, like AAV5, both the AP1 and CRE sites were functional in maintaining basal transcription of the B-AAV P41 promoter. However, the difference in activity between the B-AAV and AAV5 minCP41 constructs was significantly less than observed for the differences observed between these promoters in their native context (Fig. 3D, compare lanes 1 and 7; Fig. 3E), suggesting that genomic sequences other than those within the minimal promoter region might also contribute their different transcription profiles.

To address this possibility, we engineered two chimeric constructs based on the parent RepStop plasmids described above. The B-AAVRepStopCapAAV5P41 contains the AAV5 P41 promoter in the B-AAV background, while AAV5RepStopCapB-AAVP41 has B-AAV P41 promoter in the AAV5 background (Fig. 3C). Although the chimeric junction chosen was placed in a homologous region between the two to avoid potential disruption of Rep open reading frame, there was still the possibility of incorrect folding of the chimeric Rep protein. Therefore, a premature termination codon was introduced downstream of the P7 promoter and potential Rep functions were assayed following addition *in trans*.

The basal level of the B-AAV and AAV5 P41 promoters in the parent plasmids was again seen to be significantly different. AAV5 P41 transcription was approximately 7-fold higher than B-AAV (Fig. 3D, compare lane 1 to lane 7; Fig. 3E). This effect was not due to Rep because adding AAV5 or B-AAV Rep *in trans* did not significantly change the level of transcription (Fig. 3D, compare lanes 2 and 3 to lane 1, and lanes 8 and 9 to lane 7; Fig. 3E). However, when AAV5 P41 was placed in the B-AAV background, transcription was reduced to about half of the original level (Fig. 3D, compare lane 4 to lane 1; Fig. 3E), and Rep provided *in trans* had little additional effect (Fig. 3D, compare lanes 5 and 6 to lane 4; Fig. 3E). This level was still higher than the P41 activity observed from the wild-type B-AAV construct (Fig. 3D, lane 7; Fig. 3E), primarily because the constitutive basal activity of the AAV5 P41 promoter was greater, which was apparent in Fig. 3B. In the reciprocal experiment, in which the B-AAV P41 promoter was placed in the AAV5 background, B-AAV P41 activity was increased to about twice that of the original level (Fig. 3D, compare lane 10 to 7; Fig. 3E), although not quite to the level of the wild-type AAV5 P41 (Fig. 3D, lane 1; Fig. 3E), likely due to a lower intrinsic basal level of B-AAV P41 activity, as described above. Addition of Rep still had little effect (Fig. 3D, compare lanes 11 and 12 to lane 10; Fig. 3E). Thus, although the B-AAV P41 promoter contains functional AP1 and CRE sites like AAV5, its activity was still lower due to a combination of intrinsic properties of the promoter itself and a context-dependent effect of surrounding sequences. It remains possible that either AAV5 contains extra transcription factor binding sites in its P41 promoter or surrounding regions, or alternatively, that B-AAV may possess unidentified repressor binding sites at similar region.

B-AAV upstream transcription units in constructs with RBE-containing left-hand [1/2] ITRs are activated during Ad5 co-infection in a pattern similar to AAV2

As observed in Fig. 1, circular B-AAV and AAV5 constructs containing left-hand [1/2] ITR sequences had elevated levels of transcripts apparently originating from upstream promoters. However, in our preliminary attempts to characterize the nature of these transcripts, we found that these plasmids generated a significant level of transcripts that had "read-around" these

Fig. 3. Analysis of *cis* sequences of B-AAV and AAV5 that control P41 transcription. (A) Alignment of AAV5 (nt 1770 to nt 1970) and B-AAV (nt 1779 to 1979) P41 promoter region. Promoter constructs described below and in the text were used to drive luciferase gene expression in the reporter constructs. Consensus nucleotides are shaded. AP1 and CRE elements previously reported to mediate constitutive high level of AAV5 P41 promoter are boxed and indicated. Nucleotide numbers of core promoters (CP41, AAV5 nt 1880–1970, B-AAV nt 1889–1979) starting from TATA box, and minimum upstream sequences attached to core promoters (min CP41, starting from AAV5 nt 1770 or B-AAV nt 1779) are labeled. (B) Mutation analysis of P41 promoter. Blank bars represent the luciferase gene expression driven by various AAV5 promoters, and black bars represent luciferase expression driven by corresponding B-AAV promoters. The designation of each pair of AAV5 and B-AAV promoters is indicated below the bars (refer to Materials and methods for sequences and nucleotide numbers). The luciferase gene was driven by each core promoter sequence (CP41) starting with TATA box, or core promoters with minimum wild-type (minCP41) or AP1/CRE mutant upstream sequences (minAP1mCP41, minCREmCP41 and minAP1mCREmCP41) in 293 cells. Luciferase activity was normalized to *Renilla* luciferase (R-luc) transfection control. The values shown are the average with standard deviations from three independent plasmid transfections done in a single experiment. (C) Diagrams of wild type and chimeric RepStopCap constructs used in the assay below. Blank bar represents values for AAV5 sequences while black bar represents values for B-AAV. All constructs contain premature termination codon downstream of P7 promoter (nt 541 for B-AAV and nt 480 for AAV5). The RP probe across P41 region was used for RNase protection assay. (D) RNase protection assay of the constructs co-transfected with pBluescript SK empty-vector control (SK) or AAV5 (V) or B-AAV Rep (B) expression constructs as activators, followed by Ad5 infection. Transcription signals from GFP, P7, P19, and P41 are indicated. (E) Quantification of the protection shown in panel D. Data from at least three experiments, with standard error bars, are presented as the level of P41 transcripts normalized to GFP signal.

circular templates, potentially confounding these analyses (data not shown). To more accurately assess the activity and activation of the B-AAV and AAV5 upstream promoters, we first linearized the B-AAV and AAV5 constructs, to prevent potential read-through transcription around the circular plasmids. The RNA these plasmids generated was then assayed using separate probes across P41, P7, and P19 promoters. The latter two allowed quantification of RNAs generated individually by the P7 and P19 promoters and RNAs generated from within the ITR (ITR vs. P7 using P7 probes, ITR+P7 vs. P19 using P19 probes, Fig. 4A).

P41 transcription from both linearized B-AAV and AAV5 plasmids followed the same pattern of expression as from the circular plasmids as seen in Fig. 1, i.e., B-AAV P41 in constructs with the RBE-containing [1/2] ITR-binding site at any position was activated in the presence of Ad5 (Fig. 4B, compare lanes 20 to 19, 22 to 21 and 24 to 23; Fig. 4C bottom panel), while AAV5 P41 was not activated in any of the constructs in

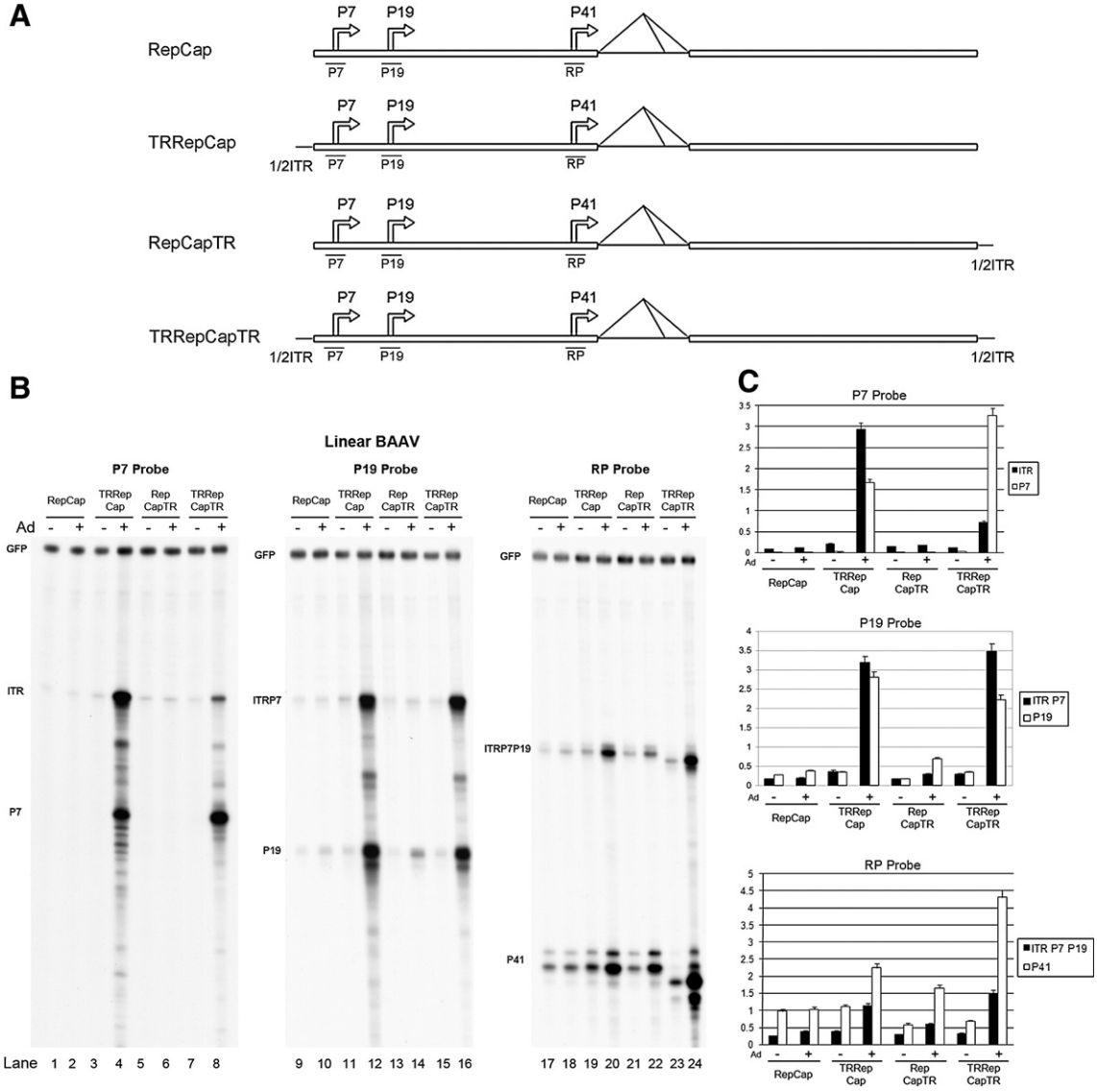


Fig. 4. Upstream transcription analysis of linearized B-AAV and AAV5 plasmids. (A) Diagrams of RepCap constructs and probes used. The RepCap constructs are the same as in Fig. 1A except they were linearized within the plasmid backbone. P7 probe spans from nt 280 to nt 440 of B-AAV and nt 278 to nt 431 of AAV5. P19 probe spans from nt 848 to nt 1005 of B-AAV and nt 839 to nt 996 of AAV5. RP probes are the same as those used in Fig. 1. (B and D) RNase protection assay following transfection of linearized version of aforementioned B-AAV and AAV5 constructs, using the P7, P19, and RP probes as described in the text. GFP probes were used as internal control. The derivation of each transcript (ITR, P7, P19, ITR+P7+P19, P7+P19, and P41) is labeled on the left side of the panel. (*) in panel D represents undigested P7 probe (as determined by co-migration with a probe sample not shown in this figure), rather than a genuine ITR transcript, which would be expected to migrate faster than the asterisk-designated band. Bands representing P41 transcription in lanes 23 and 24 (B) migrate slightly faster compared to those in other lanes due to a mismatch at the end of the RP probe which was generated during the cloning procedure, as mentioned in the legend to Fig. 1. In panel D, P19 transcripts in lanes 11, 12, 15, and 16 migrated slightly faster than those in lanes 9, 10, 13, and 14, also due to a mismatch at the end of the P19 probe generated during the cloning procedure. The nature of the extra band above the P7P19 transcript band in lane 23 is uncertain, but likely represents partially digested probe. (C and E) Quantification of the protection assays for each panel of B and D, respectively. Data from at least three experiments, with standard error bars, are presented as the level of transcripts normalized to GFP signal.

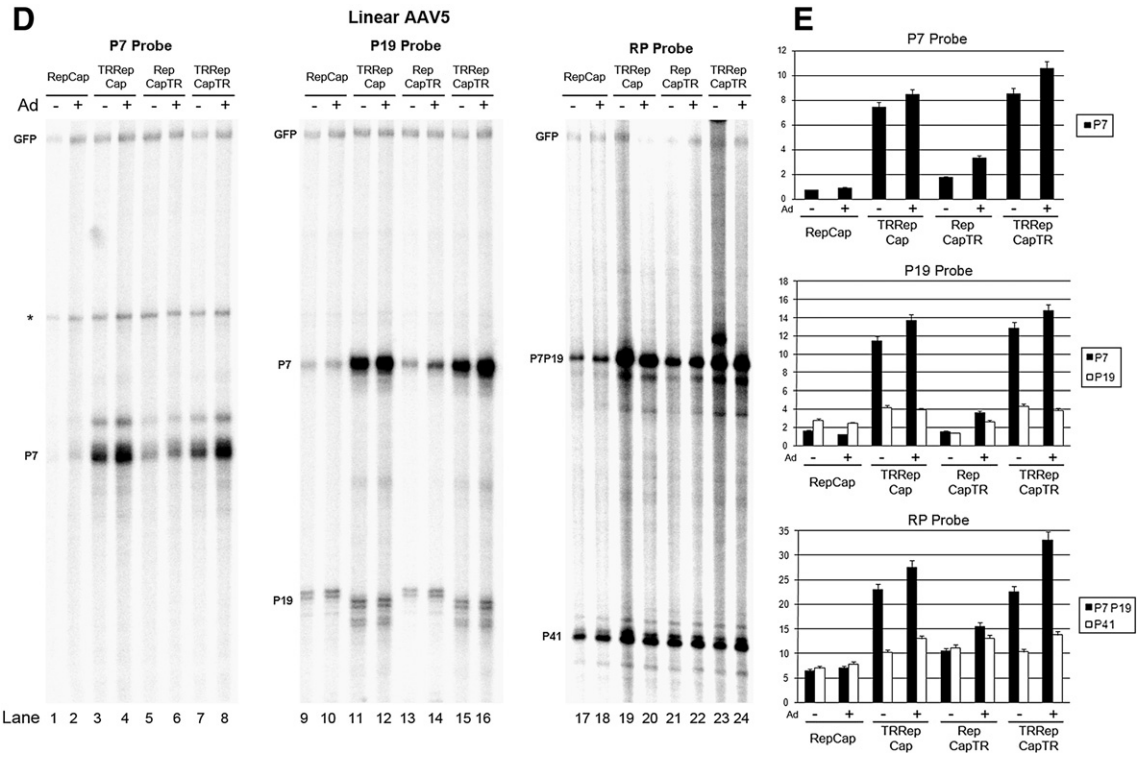


Fig. 4 (*continued*).

the same setting (Fig. 4D, compare lanes 20 to 19, 22 to 21 and 24 to 23; Fig. 4E, bottom panel). This result further confirmed that similar to AAV2, and in contrast to AAV5, the activation of B-AAV P41 by Rep requires the RBE and Ad5 helper function, and the presence of RBE is position- and orientation-independent.

Analysis of B-AAV upstream transcription revealed additional similarities to AAV2 and differences from AAV5. In constructs with a left-hand [1/2] ITR, all three transcription units (ITR, P7 and P19) were activated with Ad5 infection (Fig. 4B, lanes 4, 8, 12 and 16; Fig. 4C, top two panels), while for all the AAV5 constructs, none of the promoters showed increased transcription in response to Ad5 infection (Fig. 4D, left two panels, compare even numbered lanes with odd numbered ones; Fig. 4E, top two panels). Expression of the B-AAV and AAV5 P19 promoters was also distinctly different. In the TRRepCapTR constructs, which are likely most similar to the transcription template used during infection, B-AAV P19 activity reached levels comparable to P7 with Ad5 infection (Fig. 4B, lane 16; Fig. 4C, middle panel); however, levels of the AAV5 P19 promoter in similar constructs remained low relative to P7 transcription in the presence of Ad5 (P19:P7 approximately 1:3 in TRAAV5RepCapTR, Fig. 4D, lane 16; Fig. 4E, middle panel). This result is somewhat different from those previously observed for AAV5 virus undergoing replication in 293 cells (Qiu et al., 2002), for reasons that are currently unclear. Addition of the left-hand [1/2] ITR in the AAV5 constructs did increase levels of P7 transcription (Fig. 4D, lanes 3, 4 and lanes 7, 8; Fig. 4E, top panel); however, transcription originating from the AAV5 ITR itself was undetectable, and thus lower than that from the B-AAV ITR, which agrees with our previous observation (Qiu et al., 2006b). Thus, the increased levels of upstream-derived transcripts from the B-AAV constructs with a left-hand [1/2] ITR in the presence of Ad5 and Rep represented a combination of transcripts generated from the ITR, P7, and P19, while for AAV5, the ITR-dependent enhancement resulted in an increase only in P7 activity. Interestingly, when comparing TRB-AAVRepCap to TRB-AAV-RepCapTR, the magnitude of augmentation of the ITR and P7 promoters in response to Ad5 was different. Introduction of the right-hand [1/2] ITR reduced transcription from left-hand [1/2] ITR to approximately 1/4 of the original level, while transcription of P7 was increased about 2-fold (Fig. 4B, compare lane 8 to lane 4), a pattern which is more similar to that seen during viral infection (Qiu et al., 2006b).

Taken together, these results indicated that transcription of all three primary promoters within the B-AAV constructs with left-hand [1/2] ITR sequences present in *cis* was activated by the presence of Ad5 and Rep similarly to AAV2, while AAV5, despite its greater sequence homology with B-AAV, remained unique in this regard.

Discussion

Although we have previously reported that the overall genetic organization of B-AAV is more similar to AAV5 than to AAV2 (Qiu et al., 2006b), a more detailed characterization of B-AAV transcription has now revealed additional features that are more similar to that of AAV2, despite their lower sequence homology and different transcription maps. The basal activity of the B-AAV

P41 promoter was low compared to AAV5 P41 although it has the AP1 and CRE-binding sites that differentiate AAV5 P41 from AAV2 P40. The low activity of B-AAV P41 was due to a combination of a weak promoter itself and its surrounding genomic context, and this low level afforded a dynamic range for subsequent Rep activation. In confirmatory experiments, the linear TRB-AAVRepCapTR construct was seen to exhibit activation of its P41 capsid promoter, as well as all upstream promoters (ITR, P7 and P19) in the presence of Ad5 and Rep, while in contrast, all the transcription units (ITR, P7, P19 and P41) of the linear TRAAV5RepCapTR construct remained relatively unresponsive. Activation of the B-AAV P41 promoter by its Rep protein required an RBE, whose placement in *cis* was irrelevant; however, activation of other upstream transcription units of B-AAV required an RBE at the left-hand end to achieve its effect.

We have previously demonstrated that the AAV5 Rep protein was a poor activator of the AAV2 P40 promoter even though it bound the AAV2 P5 RBE as well as did AAV2 Rep (Ye et al., 2006). However, we have found here that AAV5 Rep is able to activate B-AAV P41 within the B-AAV background due to its low basal level in that context. Activation of the AAV5 P41 promoter by AAV5 Rep was also observed when the basal activity of AAV5 P41 promoter was decreased by mutation of its AP1 and CRE elements (Ye and Pintel, 2007). Thus, activation levels of the AAV capsid promoter are determined by both the intrinsic strength of the promoter sequences and the binding/activation properties of Rep.

In our experiments, the activation magnitude of the B-AAV capsid promoter was not directly related to the level of the large Rep-encoding P7 transcripts. In the presence of Ad5, the linear B-AAVRepCapTR construct (Fig 4B, lane 6) produced less P7 transcripts than linear TRB-AAVRepCap or TRB-AAVRepCapTR (Fig 4B, lanes 4 and 8), yet all three P41 promoters were activated to comparable levels. Similarly, activation of the AAV5 P41 promoter did not appear to be further enhanced by increased P7 transcription from constructs containing a left hand [1/2]ITR. Thus, it is likely that efficient activation of the B-AAV and AAV5 capsid gene promoters requires a relatively small threshold level of Rep, above which, little additional activation is seen. This has previously been noted for activation of the capsid gene promoter of the autonomous parvovirus minute virus of mice by its nonstructural protein NS1 (Pearson and Pintel, 2000). Such observations should be taken into account when considering how absolute levels of Rep affect the models of AAV capsid gene activation proposed below.

Based on our recent observations (Qiu and Pintel, 2002; Ye and Pintel, 2007; Ye et al., 2006), and together with previously established AAV2 transcription activation models (Pereira and Muzyczka, 1997), we summarize in Fig. 5 an overview of the capsid gene transcription strategies employed by these three different AAVs. Basal AAV2 P40 transcription activity is low in both E1A- and E1B-expressing 293 cells and HeLa cells, and this is at least partially due to the lack of AP1 and CRE-binding sites adjacent to P40, which are present in AAV5 and B-AAV P41 (Ye and Pintel, 2007). Activation relies on the AAV2 large Rep protein in the presence of helper viral functions, and Pereira et al. (Pereira and Muzyczka, 1997) have proposed that this

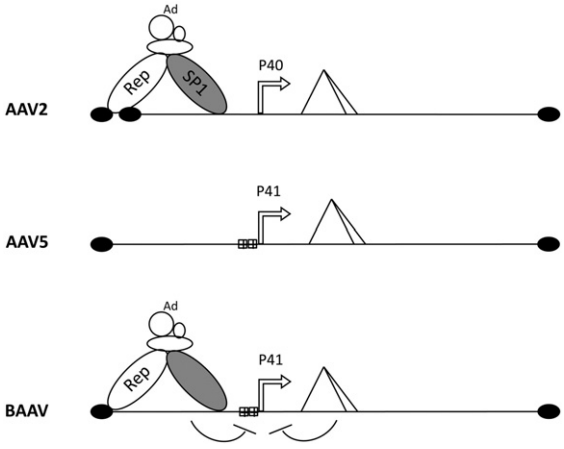


Fig. 5. Models of capsid promoter regulation of various AAVs. Arrows represent AAV2, AAV5, and B-AAV capsid promoters. Filled ovals represent Rep-binding elements (residing in ITRs at the end of each genome, and an extra one in the P5 region for AAV2). Shaded oval in the top panel represents SP1 transcription factor previously shown to interact with the AAV2 Rep protein (Pereira and Muzyczka, 1997). Adenovirus genes or unidentified cellular genes induced by adenoviral infection involved in Rep activation are shown in a cluster of empty ovals. The upstream AP1 and CRE transcription factor binding sites are in rectangles and labeled with "+". The top panel is derived from a previous study by Pereira et al. (Pereira and Muzyczka, 1997). The middle panel is based on our previous observations (Ye and Pintel, 2007), and the bottom panel is based on this study. See details in text.

activation is mediated by a loop structure formed via the interaction between AAV2 Rep, transcription factor SP1, and their respective binding sites in the viral ITR/P5 and P40 regions (Fig. 5, top panel). This structure likely helps to recruit additional transcription factors from the ITR/P5 region to the capsid promoter region thus increasing P40 transcription. On the other hand, the basal activity of the AAV5 P41 promoter in the absence of Rep is constitutively high in E1A- and E1B-expressing 293 cells (Qiu et al., 2002; Ye and Pintel, 2007), and thus Rep activation levels are less pronounced (Fig. 5, middle panel). The higher basal activity of the AAV5 P41 promoter is at least partially due to the AP1 and CRE transcription factor binding sites upstream of the P41 core promoter elements, which are lacking in AAV2 P40 (Ye and Pintel, 2007). B-AAV demonstrated a transcription profile combining the features of AAV5 and AAV2. Similar to AAV5, the B-AAV P41 promoter contains both AP1 and CRE sites upstream, which contributed to its basal activity, yet the B-AAV P41 level was still low, due to both the weak nature of the P41 sequences and a context-dependent inhibitory effect of surrounding sequences. This low basal activity allows a greater magnitude of activation by Rep (Fig. 5, bottom panel), similar to the situation for AAV2 P40, although whether activation is ultimately mediated by the same mechanism is not yet known and needs to be confirmed in the native bovine cell host. Thus, these three AAVs achieve the common goal of optimal activated levels of capsid gene RNA expression by distinct means. While many of the details of these proposed mechanisms remain unknown (including the involvement of helper virus functions), how these differences may be related to variations in the life-cycles of these viruses is interesting to consider.

Materials and methods

Cells and virus

293 cells are permissive for B-AAV replication in the presence of human Ad5 as previously described (Qiu et al., 2006b). Transfections, using Lipofectamine and the Plus reagent (Invitrogen, Carlsbad, CA), were performed as previously described (Qiu and Pintel, 2002), and when Ad5 was co-infected, this was done 5 h after transfection at a moi of 5.

Plasmid constructs

RepCap and RepStopCap plasmids

AAV5 RepCap (containing AAV5 nt 185–4448) has been described previously (Qiu et al., 2002). B-AAV RepCap (containing B-AAV nt 193–4493) was constructed by inserting B-AAV sequence between the *Sac*II–*Kpn*I sites in pBluescript SK(+) (Stratagene, La Jolla, Calif.). For AAV5, TRRepCap extends AAV5 RepCap at the 5′ end to nt 75, RepCapTR extends AAV5 RepCap at the 3′ to nt 4591, and TRRepCapTR extends both ends. For B-AAV, TRRepCap extends the 5′ end to nt 81, RepCapTR extends the 3′ end to nt 4637, and TRRepCapTR extends both ends as well. Stop codons were introduced at nt 480 in the AAV5 constructs and nt 541 in the B-AAV constructs mentioned above to make their RepStopCap derivatives. AAV5 and B-AAV Rep expression plasmids were described before (Qiu et al., 2006b, 2002); these contain silent mutations in the RP region to avoid hybridization with RNase protection probes directed against RNAs generated from the aforementioned test plasmids. The Rep proteins were expressed at comparable levels (data not shown). *Sac*II was used to linearize the AAV5 and B-AAV plasmids, except for the TRB-AAVRepCapTR plasmid, which was linearized by *Kpn*I.

Luciferase reporter plasmids

All luciferase reporter constructs are based on pGL3-Basic (Promega, Madison, WI). All AAV5 reporter constructs were mentioned previously (Ye and Pintel, 2007). B-AAV reporter constructs utilized the same cloning strategy but used B-AAV counterparts. The B-AAV core promoter (CP41) contains nt 1889–1979. B-AAVmin added B-AAV sequences (nt 1779–1888) upstream of the B-AAV CP41 construct. B-AAVmin AP1m is based on B-AAVmin with the sequence ACTCAC at nt 1797 to 1802 changed to GAGCAT. B-AAVmin CREm is based on B-AAVmin with the GT at nt 1875 to 1876 changed to TC. B-AAVminAP1mCREm contains both changes.

AAV5-B-AAV chimeric plasmids

Chimeras were based on AAV5 and B-AAV RepStopCap plasmids. The border (AAV5 nt 1472 and B-AAV nt 1481) was chosen at a significant homologous region of the two constructs. B-AAVRepStopCapAAV5P41 was based on the parent B-AAVRepStopCap plasmid with AAV5 P41 sequence (nt 1472–1974) replacing the B-AAV P41 sequence (nt 1481–1983). AAV5RepStopCapB-AAVP41 was based on the B-AAVRepStopCap parent plasmid with the reverse exchange.

RNase protection assays

Total RNA was isolated 36–41 h post-transfection as previously described (Naeger et al., 1992; Schoborg and Pintel, 1991). RNase protections were performed as previously described (Naeger et al., 1992; Schoborg and Pintel, 1991), using homologous anti-sense probes. AAV5 P7 probe spans nt 278–431, P19 probe spans nt 839–996, and RP probe spans nt 1846–1986. B-AAV P7 probe spans nt 280–440, P19 probe spans nt 848–1005, and RP probe spans nt 1855–1995.

Luciferase assays

Luciferase assays were performed according to the manufacturer's suggested protocols (Promega, Madison, WI). Briefly, 293 cells grown in 12-well plates were transfected with 0.1 μg per well of the testing luciferase reporter constructs along with 0.35 μg per well of empty pBluescript SK(+) (Stratagene, La Jolla, CA) using the Lipofectamine reagent (Invitrogen). In addition, 0.05 μg per well of thymidine kinase (TK)-driven *Renilla* luciferase gene reporter was cotransfected as an internal control. The total amount of DNA transfected into each well was kept at 0.5 μg. Thirty-six hours after transfection, cells were lysed and the luciferase activity was tested using the Promega dual-luciferase reporter assay system (Promega). Each experiment represents the average of duplicates from three individual experiments (error bars shown), each normalized to *Renilla* luciferase activity.

Acknowledgments

We thank Lisa Burger for excellent technical assistance and Jianming Qiu for helpful discussion. This work was supported by PHS grants RO1 AI46458 and RO1 AI56310 from NIAID to DJP.

References

Arbetman, A.E., Lochrie, M., Zhou, S., Wellman, J., Scallan, C., Doroudchi, M.M., Randlev, B., Patarroyo-White, S., Liu, T., Smith, P., Lehmkuhl, H., Hobbs, L.A., Pierce, G.F., Colosi, P., 2005. Novel caprine adeno-associated virus (AAV) capsid (AAV-Go.1) is closely related to the primate AAV-5 and has unique tropism and neutralization properties. J. Virol. 79 (24), 15238–15245.

Bantel-Schaal, U., Delius, H., Schmidt, R., zur Hausen, H., 1999. Human adeno-associated virus type 5 is only distantly related to other known primate helper-dependent parvoviruses. J. Virol. 73 (2), 939–947.

Bowles, D.E., Rabinowitz, J.E., Samulski, R.J., 2006. The genus Dependovirus. In: Kerr, J.R., Cotmore, S.F., Bloom, M.E., Linden, R.M., Parrish, C.R. (Eds.), Parvoviruses. Hodder Arnold, pp. 15–23.

Chiorini, J.A., Afione, S., Kotin, R.M., 1999a. Adeno-associated virus (AAV) type 5 Rep protein cleaves a unique terminal resolution site compared with other AAV serotypes. J. Virol. 73 (5), 4293–4298.

Chiorini, J.A., Kim, F., Yang, L., Kotin, R.M., 1999b. Cloning and characterization of adeno-associated virus type 5. J. Virol. 73 (2), 1309–1319.

Clarke, J.K., McFerran, J.B., McKillop, E.R., Curran, W.L., 1979. Isolation of an adeno associated virus from sheep. Brief report. Arch. Virol. 60 (2), 171–176.

Cotmore, S.F., Tattersall, P., 2006. Structure and organization of the viral genome. In: Kerr, J.R., Cotmore, S.F., Bloom, M.E., Linden, R.M., Parrish, C.R. (Eds.), Parvoviruses. Hodder Arnold, pp. 73–94.

Luchsinger, E., Strobbe, R., Wellemans, G., Dekegel, D., Sprecher-Goldberger, S., 1970. Haemagglutinating adeno-associated virus (AAV) in association with

bovine adenovirus type 1. Brief report. Arch. Gesamte Virusforsch. 31 (3), 390–392.

McCarty, D.M., Christensen, M., Muzyczka, N., 1991. Sequences required for coordinate induction of adeno-associated virus p19 and p40 promoters by Rep protein. J. Virol. 65 (6), 2936–2945.

Myrup, A.C., Mohanty, S.B., Hetrick, F.M., 1976. Isolation and characterization of adeno-associated viruses from bovine adenovirus types 1 and 2. Am. J. Vet. Res. 37 (8), 907–910.

Naeger, L.K., Schoborg, R.V., Zhao, Q., Tullis, G.E., Pintel, D.J., 1992. Nonsense mutations inhibit splicing of MVM RNA in *cis* when they interrupt the reading frame of either exon of the final spliced product. Genes Dev. 6 (6), 1107–1119.

Pearson, J.L., Pintel, D.J., 2000. Recombination within the nonstructural genes of the parvovirus minute virus of mice (MVM) generates functional levels of wild-type NS1, which can be detected in the absence of selective pressure following transfection of nonreplicating plasmids. Virology 269 (1), 128–136.

Pereira, D.J., Muzyczka, N., 1997. The adeno-associated virus type 2 p40 promoter requires a proximal Sp1 interaction and a p19 CArG-like element to facilitate Rep transactivation. J. Virol. 71 (6), 4300–4309.

Qiu, J., Pintel, D.J., 2002. The adeno-associated virus type 2 Rep protein regulates RNA processing via interaction with the transcription template. Mol. Cell. Biol. 22 (11), 3639–3652.

Qiu, J., Nayak, R., Tullis, G.E., Pintel, D.J., 2002. Characterization of the transcription profile of adeno-associated virus type 5 reveals a number of unique features compared to previously characterized adeno-associated viruses. J. Virol. 76 (24), 12435–12447.

Qiu, J., Cheng, F., Yoto, Y., Zadori, Z., Pintel, D., 2005. The expression strategy of goose parvovirus exhibits features of both the Dependovirus and Parvovirus genera. J. Virol. 79 (17), 11035–11044.

Qiu, J., Cheng, F., Pintel, D., 2006a. Molecular characterization of caprine adeno-associated virus (AAV-Go.1) reveals striking similarity to human AAV5. Virology 356 (1–2), 208–216.

Qiu, J., Cheng, F., Pintel, D.J., 2006b. Expression profiles of bovine adeno-associated virus and avian adeno-associated virus display significant similarity to that of adeno-associated virus type 5. J. Virol. 80 (11), 5482–5493.

Schoborg, R.V., Pintel, D.J., 1991. Accumulation of MVM gene products is differentially regulated by transcription initiation, RNA processing and protein stability. Virology 181 (1), 22–34.

Ye, C., Pintel, D.J., 2007. Upstream AP1- and CREB-binding sites confer high basal activity on the adeno-associated virus type 5 capsid gene promoter. J. Virol. 81 (6), 2605–2613.

Ye, C., Qiu, J., Pintel, D.J., 2006. Efficient expression of the adeno-associated virus type 5 p41 capsid gene promoter in 293 cells does not require Rep. J. Virol. 80 (13), 6559–6567.